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REMARKS

Status of the Claims

Claims 1-11, and 13-17 are pending in this application. Claim 12 has been cancelled and claims 15-17 have been added. Claims 5-10, 13 and 14, drawn to a non-elected invention, are withdrawn from consideration by the Examiner.

Claim Objections

Claim 12 is objected to by the Examiner because it does not further limit claim 11. Accordingly, claim 12 has been cancelled as being duplicative.

Rejection of Claims 11 and 12 Under 35 U.S.C. 112, First Paragraph

Claims 11 and 12 are rejected by the Examiner under 35 U.S.C. 112, first paragraph, for the reasons set forth in paragraph 6 of the Office Action. This rejection is respectfully traversed. Reconsideration and withdrawal thereof are requested.

This rejection is moot with respect to claim 12 due to the cancellation of claim 12.

The present invention as recited in claim 11 relates to a medicament for treating solid cancer comprising as a major ingredient the enzyme that produces plasma protein fragments as set forth in claim 1, that is, an aspartic enzyme that produces plasma

protein fragments having an inhibitory activity to metastasis and growth of cancer.

The Examiner's comments regarding enablement of various methods are not applicable to composition claim 11. Rather, the Examiner's comments are more accurately directed to method of treatment claims. Applicants are not required to enable all uses for a composition claim. There is no such requirement in either 35 U.S.C. 112 or in case law supporting the Examiner's position. Indeed, since the Examiner concedes that at least one use is enabled, then this single use is sufficient to satisfy the statutory requirements to support composition claim 11. Accordingly, the Examiner has failed to establish a prima facie case of nonenablement and the burden of proof has therefore not shifted to Applicants. This is especially true in the present circumstances as this rejection is entirely without foundation.

More specifically, the Examiner alleges that the medicament of claim 11 is not enabling of a method for treating all cancers and preventing disease conditions merely associated with vascularization while being enabled for a method of treating lung cancer in a mouse.

The enzyme of the present invention acts in such a way that activates an angiogenesis-inhibitory factor present in an inactivated form in blood to thereby convert the factor into an active form. The claimed enzyme does not act in a way such that it

directly acts on new blood vessels or cancer cells per se. Thus, a type of cancer or indications to be applied with the medicament of the present invention may depend upon the active angiogenesis-inhibitory factor produced by the enzyme of the present invention.

The enzyme of the present invention is only active under acidic conditions provided by cancer to thereby produce an angiogenesis-inhibitory factor. The present enzyme is not active under normal physiological conditions (i.e. at around neutral pH). Thus, the enzyme of the present invention is applicable to patients having tumors at certain stages where it may exert its activity. Most effectively, the enzyme of the present invention may be effected before proliferation of tumor (before neovascularization).

Therefore, it will be the most suitable usage of the enzyme of the present invention that it is effected when progressive cancer is first found, or after removal of primary focus by surgery for prevention of distal metastasis. Clinical techniques presently can detect cancer as small as several mm in size with techniques such as a PET scan to allow for early detection or metastasis of cancer. Although a common anti-cancer agent may also be used, from the viewpoint of adverse side effects or drug tolerance, the enzyme of the present invention may be utilized more advantageously as being free from such adverse side effects.

Even with a malignant cancer that has already developed neovascularization, the enzyme of the present invention may be

efficacious for inhibiting proliferation of tumors through inhibition of further expansion of a network of new blood vessels already formed in the tumor. See the attached sheet entitled *"Mechanism of development of neovasculanzation and network structure thereof"*.

In summary, types of cancer or applicable situations (e.g. indications) where the medicament of the present invention may be used includes:

(1) Cancer at an early stage (most effective);

(2) Use after surgery (for inhibition of proliferation of distal metastatic focus); and

(3) Effective application to cancer at a more advanced stage in combination with chemotherapy and radiotherapy.

Accordingly, the enablement rejection should be withdrawn since the Examiner has not properly set forth a *prima* facie case of nonenablement or in view of the remarks stated above. Clearly, the medicament of the present invention as recited in claim 11 is enabled for treating a solid cancer.

Rejection of Claims 1-4, 11 and 12 Under 35 U.S.C. 112, Second Paragraph

Claims 1-4, 11 and 12 are rejected by the Examiner under 35 U.S.C. 112, second paragraph, for the reasons set forth in paragraphs 7-8 of the Office Action. This rejection is respectfully traversed. Reconsideration and withdrawal thereof are requested.

The Examiner states that the phrase "[a]n enzyme that produces plasma protein fragments having an inhibitor activity" is unclear. The Examiner requests that Applicants clarify if the plasma protein fragments or the enzyme possesses the activity. Presently, the Examiner is interpreting the claim as the protein fragments having the activity.

The Examiner should note that plasma protein fragments produced through the action of the enzyme possess the inhibitory activity.

The Examiner further states that the meaning of the phrase "having a high homology" in claim 2 is vague and indefinite.

The Examiner should note that the term "high" has been canceled from claim 2. With regard to the use of the term "homology" in this context, the Examiner's attention is directed to the description at page 9, lines 17-19 of the specification, which recites "[I]t was also found that an N-terminal amino acid sequence of this enzyme was initiated with LVRIPLHKFT which had a high homology to Human Cathepsin D Precursor". Thus, the rejected recitation means **"having an N-terminal amino acid sequence homologous to Human Cathepsin D Precursor"**.

The Examiner requests clarification in claim 3 as to whether the extracellular matrix proteins are mammalian or non-mammalian proteins. The Examiner is advised that both fibronectin and vitronectin are mammalian proteins from humans.

The Examiner's position is that claim 11 is vague and indefinite due to the recitation "solid cancer". Applicants respectfully disagree.

The medicament of the present invention is efficacious for treating any type of solid cancer since proliferation is dependent upon angiogenesis (neovascularization). The plasma protein fragments that are produced by the action of the enzyme of the present invention have an inhibitory activity to metastasis and growth of cancer and may inhibit angiogenesis occurring during metastasis and cancer growth. However, metastasis and growth of cancer *per se* is not directly inhibited.

As is well known in the art, a malignant tumor (solid cancer) having a diameter of 1-3 mm or less is able to maintain its own metabolism through provision of nutrients and excretion of waste material via diffusion like normal cells. However, as the cancer proliferates, a new blood vessel (neovascularization or angiogenesis) becomes necessary for providing nutrients and for excreting waste material. In other words, cancer cells of a malignant tumor that advance to a large size are unable to proliferate without neovascularization (angiogenesis). An angiogenesis-inhibitory agent thus inhibits growth of the tumor through inhibition of angiogenesis. Thus, while a common anti-cancer agent exerts its effects directly on the cancer *per se*, an angiogenesis-inhibitory agent may be an indirect anti-cancer agent

acting through blockage of the cancer lifeline (i.e. blockage of angiogenesis or neovascularization). See attached sheet *"Mechanism of development of neovascularization and network structure thereof"*.

Action of an angiogenesis-inhibitory agent is summarized as follows:

(1) Blockage of lifelines providing nutrients and removing waste material from the cancer cells, thus leading to inhibition of cancer cell proliferation;

(2) Blockage of the introduction of a new blood vessel from an existing blood vessel by a minute cancer, thus leading to preventive treatment; and

(3) Inhibition of a network structure formation of blood vessels within lumps of cancer cells, thus leading to treatment.

As such, any cancer whose proliferation is dependent upon formation of a new blood vessel (i.e. angiogenesis or neovascularization), for instance, any solid cancer such as lung, breast, colon, or liver cancer, irrespective of its location in the living body, may be treated by the medicament of the present invention. On the contrary, a liquid cancer such as myeloma is not expected to be efficaciously treated by the medicament of the present invention.

Accordingly, this rejection under 35 U.S.C. 112, second paragraph, should be withdrawn in view of the remarks hereinabove.

Rejection of Claims 1-4, 11 and 12 Under 35 U.S.C. 102(b)

Claims 1-4, 11 and 12 are rejected by the Examiner under 35 U.S.C. 102(b), for the reasons set forth in paragraphs 9-10 of the Office Action. This rejection is respectfully traversed. Reconsideration and withdrawal thereof are requested.

The Examiner indicates that although Gately does not specifically recite the molecular weight, the N-terminal amino acid sequence, the SEQ ID NO: 1 and at what pH range the plasma protein fragments are degraded, these limitations are "inherent".

Contrary to the position taken by the Examiner, the enzyme of the present invention is quite distinct from the enzyme of Gately et al. The enzyme of the present invention as recited in claim 1 is an **aspartic** enzyme that produces protein fragments having an inhibitory activity to metastasis and growth of cancer. As recited in claim 2, the claimed enzyme has a molecular weight of about 45 kDa having an N-terminal amino acid sequence homologous to human cathepsin D precursor and that acts at an acidic pH range of pH 5.0 or less (e.g. not more than pH 5.0) to produce protein fragments from plasminogen. These fragments are angiostatin-like molecule(s) with a molecular weight of 40 or 43 kDa comprising Kringle 1 to Kringle 4 (see claim 15).

On the other hand, the enzyme of Gately et al. is a **serine** protease, acting only at neutral pH but not acting at an acidic pH range, that produces an angiostatin-like molecule with a molecular weight of about 50 kDa comprising Kringle 1 to Kringle 4 and part of Kringle 5.

In order to further prove the distinction between the enzyme of the present invention and the enzyme of Gately et al., Applicants will file a Rule 132 Declaration upon receipt by the undersigned.

It is true that the present invention has been completed starting from a study of Gately et al. For example, see pages 13-16 of the specification. In fact, the present inventors recognized the presence of an angiostatin-producing activity in PC-3 culture supernatant as reported by Gately et al. It is also true that the present inventors tried to purify an enzyme responsible for said enzymatic activity earlier than Gately et al.

However, as described in the specification, the present inventors' view was that the enzymatic activity reported by Gately et al. (the enzyme per se had not yet been isolated and identified at the time) is not likely to have angiostatin producing activity as Gately et al. later found that plasmin and free cysteine donors were responsible for the enzymatic activity (Gately et al., Proc. Natl. Acad. Sci. USA, Vol. 94, pp.10868-10872 (1997)).

Contrary to disclosure of Gately et al., the present inventors have successfully found, in addition to an enzyme that fragments plasminogen at neutral pH, that an enzyme activity that can specifically cleave a restricted site of plasminogen under a lower pH condition was present in PC-3 culture supernatant. This enzymatic activity was not previously reported and is utterly different from the enzymatic activity of Gately et al.

The most important distinction between the enzymatic activity of Gately et al. and the enzyme of the present invention is a pH range at which an enzymatic activity may be exerted. See claim 16.

Fig. 1 of the instant application indicates that fragmentation patterns of plasminogen by PC-3 culture supernatant, which culture supernatant is identical to that used by Gately et al. with respect to the source of the cells, culture medium and culture conditions, vary with a pH range at which the PC-3 culture supernatant is placed. Specifically, at around neutral pH where a serine protease is active, appearance of angiostatin-like protein, as reported by Gately et al., was detected at a corresponding molecular weight. The present inventors have also detected the enzymatic activity as reported by Gately et al. On the other hand, when pH is shifted to an acidic range, the activity to produce said angiostatin-like protein disappears. Instead, a distinct angiostatin-like protein thought to be a cleaved product by a distinct acidic protease is

detected at a different molecular weight from that of Gately et al. This finding at an acidic pH range for the enzymatic activity and the cleaved product therefrom is that of the present invention, i.e. an aspartate protease according to the present invention (PACE4) and an angiostatin-like protein obtained from plasminogen with said enzyme.

The present inventors have focused on enzymatic activity that is only active at an acidic pH range to cleave plasminogen to produce angiostatin-like protein so that an enzyme responsible for said enzymatic activity may be purified and identified, which enzyme however is distinct from the enzymatic activity of Gately et al.

Although Gately et al. do not expressly mention at which pH the enzymatic activity is detected, it should be noted that the enzymatic activity of Gately et al. is inhibited by a serine proteinase inhibitor but not by an aspartic proteinase inhibitor. Table 1 demonstrates that the Gately et al. enzymatic activity is of a serine protease. In this regard, it should also be noted that a serine protease works only at a neutral pH range but not at an acidic pH range, e.g. pH 3 or 4, due to the electric charge of serine at the active center. Table 1 of Gately et al. indicates that only serine proteinase inhibitors blocked angiostatin generation but none of the other classes of proteinase inhibitors including an aspartate proteinase inhibitor such as pepstatin were

effective (see bridging paragraph between pages 4888 and 4889). In other words, Gately et al. failed to detect the presence of any enzymatic activity that could be blocked by an aspartate proteinase inhibitor, i.e. the presence of an aspartate protease like PACE4 according to the present invention.

Contrary to the teachings of Gately et al., the enzyme of the present invention is an aspartate protease that is inhibited by an aspartic protease inhibitor and works only at an acidic pH range (page 9, lines 19-21 of the specification). See claim 17.

With the knowledge that the enzymatic activity of Gately et al. is of a serine protease and that a serine protease is only active at a neutral pH range, one of ordinary skill in the art would also not have been motivated to investigate whether any acidic protease like the enzyme of the present invention is present in PC-3 culture supernatant. The present inventors, however, recognized the possibility of the presence of such an enzyme with an angiostatin-like activity that acts at an acidic pH range as described in the specification, thus leading to the present invention.

In summary, the enzyme of the present invention is **an aspartate protease acting at an acidic pH range** whereas the enzymatic activity of Gately et al. is **a serine protease acting at a neutral pH range** such that the former is inhibited by an aspartate protease inhibitor while the latter by a serine protease

inhibitor. It should be noted that an aspartate protease and a serine protease are quite distinct enzymes from each other as a matter of course.

As a consequence of the difference between the enzyme of the present invention and the enzymatic activity of Gately et al., plasminogen is cleaved at different sites of the amino acid sequence thereof to produce different cleaved products, in this case angiostatin-like proteins. More specifically, the enzyme of the present invention produces from plasminogen an angiostatin-like molecule with a molecular weight of 40 or 43 kDa comprising Kringle 1 to Kringle 4 whereas the enzymatic activity of Gately et al. produces an angiostatin-like molecule with a molecular weight of about 50 kDa comprising Kringle 1 to Kringle 4 and part of Kringle 5. This was subsequently demonstrated by P. Stathakis et al. *(The Journal of Biological Chemistry, Vol. 274, No. 19, p. 8910-8916,* 1999; a copy enclosed). These two products are distinct from each other but happen to have the similar activity, i.e. an activity to inhibit angiogenesis.

A Comparison between the enzymes of the present invention and Gately et al. is summarized in the attached sheet entitled "*Comparison between the enzymes of the present invention and of Gately* et *al.*".

Accordingly, this prior art rejection should be withdrawn in view of the remarks hereinabove.

Conclusion

Pursuant to 37 C.F.R. §§ 1.17 and 1.136(a), Applicant(s) respectfully petition(s) for a one (1) month extension of time for filing a reply in connection with the present application, and the required fee of $110.00 is attached hereto.

Should there be any outstanding matters that need to be resolved in the present application, the Examiner is respectfully requested to contact Marc S. Weiner (Reg. No. 32,181) at the telephone number of the undersigned below, to conduct an interview in an effort to expedite prosecution in connection with the present application.

If necessary, the Commissioner is hereby authorized in this, concurrent, and future replies, to charge payment or credit any overpayment to Deposit Account No. 02-2448 for any additional fees required under 37 C.F.R. §§ 1.16 or 1.17; particularly, extension of time fees.

Respectfully submitted,

BIRCH, STEWART, KOLASCH & BIRCH, LLP

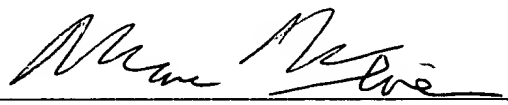
By 

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Table: Comparison between the enzymes of the present invention and of Gately et al.

	Gately et al.	Present Invention
Type of enzyme	• **Serine** proteinase (Only serine proteinase inhibitors block angiostatin generation; p. 4888, col. 2, l. 41 - p. 4889, col. 1, l. 1 & p. 4889, Table 1)	• **Aspartic** proteinase (p. 9, l. 19-21) • activity of the enzyme of the present invention was completely inhibited by pepstatin A, a specific inhibitor to an aspartic enzyme (p. 19, l. 14-20 & Fig. 5)
pH condition where the enzyme is active	• no description	• the enzyme of the present invention fragments plasminogen only at **an acidic range of pH** (p. 19, l. 22-24 & Fig. 6) • distinct fragmentation patterns of plasminogen were apparent between pH ranges of more and less than 5.0 (Example 3, p. 39, l. 2-4 & Fig 1)
N-terminal amino acid sequence of the enzyme	• no description	• this enzyme was initiated with LVRIPLHKFT which had a high homology to Human Catepcin D Precursor (p. 9, l. 17-19)
MW of the enzyme	• no description	• about 45kDa (p. 9, l. 15-16)
Plasma protein fragment	• **Fragmentation of Plasminogen at Kringle 5** • Molecular Weight (angiostatin): about 50kDa (p. 4888, col. 2, l. 12 - 15 & Fig. 1A)	• a fragment comprising **Kringles 1 to 4** produced by PACE4 (p. 19, l. 24-p. 20, l. 3 & Fig. 6) • Molecular Weight: 40kDa, 43kDa (Example 4, p. 39, l. 18-19 & Fig. 11)
Other features	• angiostatin at 10 μ/ml completely inhibited the bFGF-induced angiogenic response (p. 4889, col. 1 l. 44-col. 2, l. 9)	• the Catepsin D precursor in the form of a precursor does cleave plasminogen (p. 23, l. 20-23) • also cleaves plasmin in the vicinity its active center (between the 699th Phe and 700th Ala) to let plasmin be inactivated (p. 20, l. 25 - p. 21, l. 4)

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Angiostatin Formation Involves Disulfide Bond Reduction and Proteolysis in Kringle 5 of Plasmin*

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Plasmin is processed in the conditioned medium of HT1080 fibrosarcoma cells producing fragments with the domain structures of the angiogenesis inhibitor, angiostatin, and microplasmin. Angiostatin consists of kringle domains 1–4 and part of kringle 5, while microplasmin consists of the remainder of kringle 5 and the serine protcinase domain. Our findings indicate that formation of angiostatin/microplasmin involves reduction of plasmin by a plasmin reductase followed by proteolysis of the reduced enzyme. We present evidence that the Cys^{461}–Cys^{540} and Cys^{511}–Cys^{535} disulfide bonds in kringle 5 of plasmin were reduced by plasmin reductase. Plasmin reductase activity was secreted by HT1080 and Chinese hamster ovary cells and the human mammary carcinoma cell lines MCF-7, MDA231, and BT20 but not by the monocyte/macrophage cell line THP-1. Neither primary foreskin fibroblasts, blood monocyte/macrophages, nor macrovascular or microvascular endothelial cells secreted detectable plasmin reductase. In contrast, cultured bovine and rat vascular smooth muscle cells secreted small but reproducible levels of plasmin reductase. Reduction of the kringle 5 disulfide bonds triggered cleavage at either Arg^{529}-Lys^{530} or two other positions C-terminal of Cys^{461} in kringle 5 by a serine proteinase. Plasmin autoproteolysis could account for the cleavage, although another proteinase was mostly responsible in HT1080 conditioned medium. Three serine proteinases with apparent M_r of 70, 50, and 39 were purified from HT1080 conditioned medium, one or more of which could contribute to proteolysis of reduced plasmin.

The formation of new blood vessels from preexisting vessels is an important factor in a broad spectrum of diseases (1). New blood vessel growth by the process of angiogenesis is balanced by several protein activators and inhibitors. One such inhibitor is angiostatin, which accumulated in the murine circulation in the presence of a growing Lewis lung tumor and disappeared when the tumor was removed (2). The angiostatin produced by the primary tumor was found to inhibit the neovascularization and growth of its remote metastases. Angiostatin has been shown to inhibit the growth of a number of murine and human primary carcinomas in mice (3–5). The mechanism of action of angiostatin is not known but may relate to the induction of endothelial cell apoptosis (6).

Angiostatin is an internal fragment of plasminogen consisting of approximately the first four kringle domains.[1] Both metalloproteinase and serine proteinase activity have been implicated in the formation of angiostatin. Angiostatin fragments are generated from plasminogen by metalloelastase (7), MMP-7[2] (8), MMP-9 (8), and MMP-3 (9). Dong *et al.* (7) proposed that angiostatin is produced by metalloelastase secreted by tumor-infiltrating macrophages. Serine proteinase activity was required for the generation of angiostatin from plasminogen or plasmin by cultured human prostate carcinoma cells (10), and generation of angiostatin from plasmin by Chinese hamster ovary (CHO) or HT1080 human fibrosarcoma cells (11).

Production of angiostatin by CHO or HT1080 cells involves reduction of one or more disulfide bonds in plasmin followed by proteolysis of the reduced enzyme by a serine proteinase (11). The plasmin disulfide bond(s) are reduced by a secreted reductase, which we have called plasmin reductase. Plasmin reductase requires a small cofactor for activity, and physiologically relevant concentrations of reduced glutathione or cysteine fulfill this role. Angiostatin can also be generated from plasmin with the reductants, thioredoxin (11), protein disulfide isomerase (11), or high concentrations of small thiols (12).

In this study, we present evidence that the Cys^{461}–Cys^{540} and Cys^{511}–Cys^{535} disulfide bonds in kringle 5 of plasmin were reduced by plasmin reductase. Plasmin reductase activity was secreted by the transformed cell lines, HT1080, CHO, MCF-7, MDA231, and BT20, but not by the monocyte/macrophage cell line, THP-1. Cultured bovine and rat vascular smooth muscle cells secreted small but reproducible levels of plasmin reductase, but neither primary foreskin fibroblasts, blood monocyte/macrophages, nor macrovascular or microvascular endothelial cells secreted detectable plasmin reductase. Reduction of the kringle 5 disulfide bonds triggered cleavage at either Arg^{529}-Lys^{530} or two other positions C-terminal of Cys^{461} in kringle 5 by a serine proteinase. Three serine proteinases were purified from HT1080-conditioned medium (HT1080cm), one or more of which could account for proteolysis of reduced plasmin.

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[1] Angiostatin has been loosely defined in the literature as an internal fragment of plasminogen consisting of kringles 1–4 or smaller fragments thereof. We define angiostatin as a protein consisting of kringles 1–4 and part of kringle 5 of plasmin.

[2] The abbreviations used are: MMP, matrix metalloproteinase; CHO, Chinese hamster ovary K-1; HT1080cm, HT1080 conditioned medium; MPB, 3-(*N*-maleimidylpropionyl)biocytin; PVDF, polyvinylidene difluoride; PAGE, polyacrylamide gel electrophoresis; SBTI, soybean trypsin inhibitor; ELISA, enzyme-linked immunosorbent assay; MES, 4-morpholineethanesulfonic acid.

This paper is available on line at http://www.jbc.org

EXPERIMENTAL PROCEDURES

Chemicals and Proteins—3-(*N*-maleimidylpropionyl)biocytin (MPB) was purchased from Molecular Probes (Eugene, OR), while soybean trypsin inhibitor (SBTI) and SBTI-agarose were from Sigma-Aldrich (Sydney, Australia). Plasminogen was purified from fresh frozen human plasma and separated into its two carbohydrate variants according to published procedures (13). Glu¹-plasminogen was used in the experiments described herein. Urokinase plasminogen activator was a gift from Serono Australia. Plasmin was generated by incubating plasminogen (20 μM) with urokinase plasminogen activator (20 nM) for 30 min in 20 mM Hepes, 0.14 M NaCl, pH 7.4 buffer at 37 °C. Val-Phe-Lys-chloromethyl ketone was from Calbiochem. Plasmin (20 μM) was inactivated by incubation with Val-Phe-Lys-chloromethyl ketone (40 μM) for 60 min at room temperature. The treated plasmin contained <0.01% active plasmin measured using hydrolysis of H-D-Val-Leu-Lys-*p*-nitroanilide (S2251; Kabi, Mölndal, Sweden). Plasminogen fragments were generated by limited proteolysis with porcine elastase and purified by a combination of lysine-Sepharose affinity and Sephacryl S-100 gel filtration chromatography (Amersham Pharmacia Biotech, Uppsala, Sweden) as described previously (14). Miniplasmin was generated by incubating miniplasminogen (2 μM) with urokinase plasminogen activator (20 nM) for 2 h in 20 mM Hepes, 0.14 M NaCl, pH 7.4 buffer at 37 °C. Thioredoxin-derived angiostatin fragments were prepared as described previously (11). Protein concentrations were determined using the Bio-Rad protein assay kit and plasminogen as the standard. All proteins were aliquoted and stored at −80 °C until use.

Cell Culture—Human foreskin fibroblasts (15), blood monocyte/macrophages (16), human umbilical vein (17), human dermal microvascular (18) and bovine aortic endothelial (19) cells, and rat vascular smooth muscle cells (20) were harvested and cultured as indicated. Bovine vascular smooth muscle cells were purchased from Cell Applications (San Diego, CA). HT1080, CHO-K1, MCF-7, BT20, MDA231, and THP-1 cells were purchased from American Type Cell Culture (Rockville, MD). All media components were from Life Technologies, Inc. Conditioned medium was collected by incubating cells at ~80% confluence with Hanks' balanced salt solution containing 25 mM Hepes at pH 7.4 for 6 h. The ratio of number of cells to volume of conditioned medium was 1-3 × 10^6 cells/ml. All conditioned medium was passed through a 0.22-μm filter prior to storage at −80 °C.

Angiostatin Generation—Conditioned medium (1 ml) was incubated with plasmin (10 μg) for 2 h at 37 °C. Angiostatin fragments were labeled with MPB (100 μM) for 30 min at room temperature, followed by quenching of the unreacted MPB with GSH (200 μM) for 10 min at room temperature. Unreacted GSH and other free sulfhydryls in the system were blocked with iodoacetamide (400 μM) for 10 min at room temperature. The plasmin kringle products were collected on 50 μl of packed lysine-Sepharose beads by incubation on a rotating wheel for 1 h at room temperature; washed three times with 20 mM Hepes, 0.14 M NaCl, pH 7.4 buffer; and eluted with 50 mM ε-aminocaproic acid in the Hepes buffer.

Purification of Angiostatin from HT1080cm—Angiostatin was generated from 1 mg of plasmin in 100 ml of HT1080cm as described above. The conditioned medium was applied to a 1 × 20-cm column of lysine-Sepharose (Amersham Pharmacia Biotech,), and the matrix was washed with 20 mM Hepes, 0.14 M NaCl, pH 7.4 buffer. The bound proteins were eluted with a linear gradient of ε-ACA to 12 mM in the Hepes buffer. The angiostatin fragments were separated from a small amount of aggregated protein by gel filtration on a 2 × 50-cm Sephacryl S-100 (Amersham Pharmacia Biotech,) column in the Hepes buffer.

Electrophoresis and Western Blotting—Samples were separated on 10 or 15% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) under nonreducing or reducing conditions (21), transferred to PVDF membrane, and developed and visualized using chemiluminescence according to the manufacturer's instructions (DuPont). Both rabbit anti-human plasminogen polyclonal antibodies and swine anti-rabbit IgG horseradish peroxidase-conjugated antibodies (Dako, Carpinteria, CA) were used at 1:2000 dilution. Streptavidin-horseradish peroxidase (Amersham Australia, Sydney, Australia) was used at a 1:2000 dilution.

ELISA for MPB-labeled Angiostatin—NeutraLite avidin (Molecular Probes, Eugene, OR) (100 μl of 5 μg/ml in 15 mM Na_2CO_3, 35 mM $NaHCO_3$, 0.02% azide, pH 9.6) were adsorbed to Nunc PolySorp 96-well plates (Nunc, Roskilde, Denmark) overnight at 4 °C in a humid environment. Wells were washed once with 20 mM Hepes, 0.14 M NaCl, pH 7.4 buffer containing 0.05% Tween 20 (Hepes/Tween). Nonspecific binding sites were blocked by adding 200 μl of 2% bovine serum albumin in 15 mM Na_2CO_3, 35 mM $NaHCO_3$, 0.02% azide, pH 9.6 buffer and incubating for 90 min at 37 °C, and then wells were washed two times with Hepes/Tween. MPB-labeled angiostatin fragments were diluted in Hepes/Tween, and 100-μl aliquots were added to avidin-coated wells and incubated for 30 min at room temperature with orbital shaking. Wells were washed three times with Hepes/Tween and 100 μl of 5 μg/ml murine anti-K1–3 monoclonal antibody (American Diagnostica, Greenwich, CT) added and incubated for 30 min at room temperature with orbital shaking. Wells were washed three times with Hepes/Tween, and rabbit anti-mouse IgG horseradish peroxidase-conjugated antibody was added at a 1:500 dilution in 100 μl of Hepes/Tween and incubated for 30 min at room temperature with orbital shaking. Wells were washed three times with Hepes/Tween, and the color was developed with 100 μl of 0.003% H_2O_2, 1 mg/ml 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) in 50 mM citrate, pH 4.5 buffer for 20 min at room temperature with orbital shaking. Absorbances were read at 405 nm using a Molecular Devices Thermomax Kinetic Microplate Reader (Molecular Devices Corp., Palo Alto, CA). Results were corrected for control wells not incubated with MPB-labeled angiostatin.

Gelatin Zymography—Gelatin zymography was a modification of the technique originally described by Heussen and Dowdle (22). Bovine type B gelatin (Sigma-Aldrich) was incorporated into a 10% SDS-polyacrylamide gel at a final concentration of 1 mg/ml. Following electrophoresis, gels were washed twice with 20 mM Hepes, 0.14 M NaCl, pH 7.4 buffer containing 2.5% Triton X-100 for 30 min to remove the SDS. Gels were then incubated in 20 mM Hepes, 0.14 M NaCl, pH 7.4 buffer overnight at 37 °C and stained with Coomassie Brilliant Blue. On some occasions, gels were incubated with 20 mM Hepes, 0.14 M NaCl, pH 7.4 buffer containing 10 mM EDTA to inactivate metalloproteinases in the HT1080cm.

Plasmin Amidolytic Activity—Plasmin (10 μg) was incubated with HT1080cm (1 ml) at 37 °C. At discrete time intervals, aliquots (20 μl) of the reactions were diluted 10-fold into 20 mM Hepes, 0.14 M NaCl, 1 mg/ml PEG 6000, pH 7.4 buffer containing 200 μM H-D-Val-Leu-Lys-*p*-nitroanilide. The initial rates of release of *p*-nitroaniline from the chromogenic substrate were measured as described previously (23).

Purification of Serine Proteinases from HT1080cm—Conditioned medium (200 ml) from HT1080 cells was concentrated 20-fold over a 10-kDa cut-off membrane (Amicon, Beverly, MA) and loaded onto a 10-ml SBTI-agarose column (Sigma-Aldrich) equilibrated with 20 mM Hepes, 0.14 M NaCl, pH 7.4 buffer. The SBTI-agarose was washed with two column volumes of Hepes buffer containing 1 M NaCl and re-equilibrated with Hepes buffer containing 0.14 M NaCl. The serine proteinases were eluted from the column with 10 mM MES, 10 mM Hepes, pH 5.2 buffer containing 0.8 M benzamidine. The eluate was extensively dialyzed against 20 mM Hepes, 0.14 M NaCl, pH 7.4 buffer and stored at −80 °C until use.

Quantitation of GSH in HT1080cm—HT1080 cells at ~80% confluence were washed twice with PBS and incubated with Hanks' balanced salt solution containing 25 mM Hepes at pH 7.4 for 6 h (0.7 × 10^6 cells/ml of medium). Conditioned medium was passed through a 0.22-μm filter prior to storage at −80 °C. The low M_r thiol compounds in the conditioned medium were derivatized with the fluorescent compound 7-benzo-2-oxa-1,3-diazole-4-sulfonic acid and resolved by reverse-phase high performance liquid chromatography as described previously (24). GSH and GSSG levels were determined as described by Vandeputte *et al.* (25).

RESULTS

Angiostatin Fragments Produced in HT1080cm—Stathakis *et al.* (11) observed that three angiostatin fragments were made in CHO or HT1080cm with apparent M_r of 45, 41, and 38. These fragments were generated in HT1080cm, labeled with MPB, and purified by lysine-Sepharose affinity and gel filtration chromatography. Accordingly, three fragments were purified with apparent M_r of 45, 41, and 38 on nonreducing SDS-PAGE (Fig. 1*A*). The apparent M_r of the three fragments on reducing SDS-PAGE were 66, 60, and 57 (not shown). All three fragments were labeled with MPB, indicating that all fragments contained free thiols. Three angiostatin fragments with similar M_r were also generated by incubation of plasmin with reduced thioredoxin as previously reported (Fig. 1*B*) (11).

ELISA for MPB-labeled Angiostatin—To estimate secretion of plasmin reductase by cultured cells, an ELISA assay for MPB-labeled angiostatin was developed. This assay measured angiostatin generated by plasmin reductase and, therefore, was a relative measure of plasmin reductase activity. Briefly,

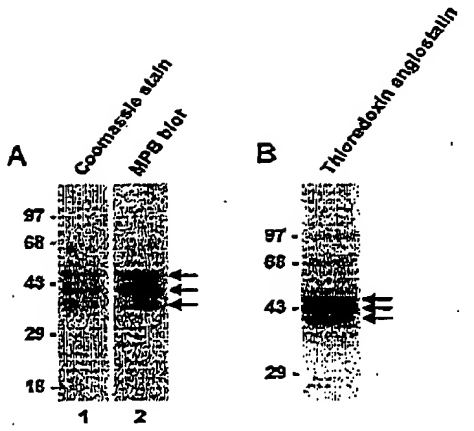

FIG. 1. **Angiostatin generated in HT1080cm or by thioredoxin.** *A*, plasmin (10 μg/ml) was incubated in HT1080cm for 24 h at 37 °C, and the angiostatin fragments were labeled with MPB. Angiostatin fragments were purified from HT1080cm by lysine-Sepharose affinity and Sephacryl S-200 gel filtration chromatography, and 2 μg was resolved on nonreducing 10% SDS-PAGE and stained with Coomassie Blue (*lane 1*) or transferred to PVDF membrane and blotted with streptavidin peroxidase to detect the MPB label (*lane 2*). The positions of M_r markers are shown at the *left*. *B*, thioredoxin-derived angiostatin fragments (2 μg) resolved on nonreducing 10% SDS-PAGE and stained with Coomassie Blue (11). The positions of M_r markers are shown at the *left*.

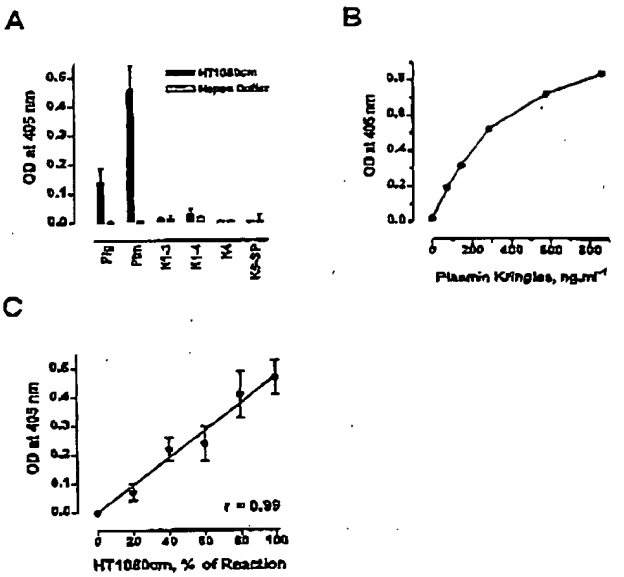

FIG. 2. **ELISA for MPB-labeled angiostatin.** *A*, plasminogen, plasmin, K1–3, K1–4, K4, or K5-serine proteinase (10 μg/ml) was incubated in either 20 mM Hepes, 0.14 M NaCl, pH 7.4 buffer (*open bars*) or HT1080cm (*solid bars*) for 2 h at 37 °C, and the angiostatin fragments were labeled with MPB. Plasmin fragments were collected on lysine-Sepharose and eluted with 20 mM ε-aminocaproic acid. The MPB-labeled angiostatin fragments were immobilized on avidin-coated wells and detected using a K1–3 monoclonal antibody and a peroxidase-conjugated anti-mouse IgG secondary antibody. *B*, plasmin (10 μg/ml) was incubated in HT1080cm, and the angiostatin fragments were labeled with MPB and collected on lysine-Sepharose as described for *A*. The concentration of plasmin kringle fragments was determined by protein assay. The *figure* shows the concentration dependence of plasmin kringle fragments in the ELISA. *C*, plasmin (10 μg/ml) was incubated in 20 mM Hepes, 0.14 M NaCl, pH 7.4 buffer and increasing HT1080cm such that the fraction of HT1080cm varied from 0 to 100% of the incubation volume. The reaction was incubated for 2 h at 37 °C, and the angiostatin fragments were labeled with MPB and detected by ELISA as described for *A*. The *solid line* represents the linear regression fit of the data ($r = 0.99$). The *bars* represent the mean and range of duplicate experiments.

plasmin was incubated with conditioned medium, and the angiostatin fragments were labeled with the biotin-linked maleimide, MPB. The MPB-labeled angiostatin fragments were adsorbed to avidin-coated microtiter plate wells, and the bound angiostatin was detected using a murine kringle 1–3 monoclonal antibody and a secondary peroxidase-conjugated antibody.

To test the specificity of the ELISA, plasminogen, plasmin, or the plasminogen fragment kringles 1–3 (K1–3), K1–4, K4, or K5-serine proteinase (10 μg/ml) were incubated in either Hepes-buffered saline or HT1080cm for 2 h at 37 °C, and the angiostatin fragments were labeled with MPB and quantitated by ELISA. The plasminogen fragments were prepared by limited proteolytic digestion of plasminogen with porcine elastase and purified by a combination of lysine-Sepharose affinity and gel filtration chromatography (14). MPB-labeled angiostatin fragments were only produced in HT1080cm from plasmin or plasmin derived endogenously from plasminogen (11) (Fig. 2*A*). As anticipated, no MPB-labeled angiostatin fragments derived from the plasminogen fragments in HT1080cm. Also, no MPB-labeled angiostatin fragments were produced from incubation of plasminogen, plasmin, or any of the plasminogen fragments in Hepes-buffered saline for 2 h at 37 °C. This result served as a negative control for plasmin reductase. The response of the ELISA was linear up to a plasmin kringle fragment concentration of ~200 ng/ml (Fig. 2*B*).

It is important to note that the ELISA assay was not an absolute measure of angiostatin formation. It is possible that one or more of the free thiols on angiostatin were refractive or inefficiently labeled by MPB due to steric factors or that two thiols on a proportion of the angiostatin molecules oxidized to form an intra- or interchain disulfide bond, which was not labeled with MPB. These considerations would have resulted in underestimation of the angiostatin generated. Nevertheless, the ELISA was a relative measure of angiostatin formation or plasmin reductase activity in serum-free conditioned medium. For example, generation of MPB-labeled angiostatin was a linear function of the concentration of HT1080cm in the reaction or of plasmin reductase concentration ($r = 0.99$) (Fig. 2*C*).

Plasmin Reductase Activity Secreted by Selected Primary and Transformed Cells—Conditioned medium from selected primary and transformed cells was collected by incubating cells at ~80% confluence with Hanks' balanced salt solution containing 25 mM Hepes, pH 7.4, buffer for 6 h. The ratio of number of cells to volume of conditioned medium was between 1 and 3 × 10^6 cells/ml. Plasmin (10 μg/ml) was incubated with the conditioned medium, and MPB-labeled angiostatin fragments were quantitated by ELISA (see Fig. 2). ELISA results were corrected for background angiostatin formation in unconditioned medium, which was negligible.

Neither human foreskin fibroblasts, bovine aortic endothelial cells, human umbilical vein endothelial cells, nor human dermal microvascular endothelial cells secreted plasmin reductase activity (Fig. 3). However, cultured rat or bovine vascular smooth muscle cells secreted plasmin reductase and converted plasmin to angiostatin, although the activity was 6% of the reductase activity secreted by HT1080 cells. CHO and MCF-7 cells secreted approximately 60%, BT20 cells 27%, and MDA231 cells 20% of the plasmin reductase activity secreted by HT1080 cells. THP-1 cells did not secrete detectable levels of plasmin reductase.

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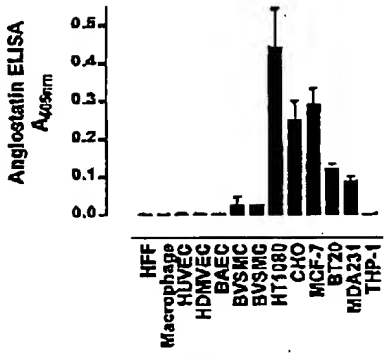

FIG. 3. **Plasmin reductase activity secreted by selected primary and transformed cells.** Conditioned medium from selected primary and transformed cells was collected by incubating cells at ~80% confluence with Hanks' balanced salt solution containing 25 mM Hepes at pH 7.4 for 6 h. The ratio of number of cells to volume of conditioned medium was 1–3 × 10^6 cells/ml. Plasmin (10 μg/ml) was incubated with conditioned medium for 2 h, and angiostatin formation was quantitated by ELISA (see Fig. 2). The *bars* represent the mean and range of duplicate experiments.

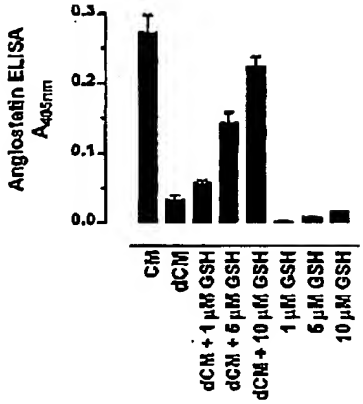

FIG. 4. **Cofactor requirements of plasmin reductase.** HT1080cm was dialyzed for 16 h against 20 mM Hepes, 0.14 M NaCl, pH 7.4, buffer using a 12–14-kDa cut-off membrane. Plasmin (10 μg/ml) was incubated in the dialyzed conditioned medium (*dCM*) or Hepes buffer for 2 h in the absence or presence of 0, 1, 5, or 10 μM GSH, and angiostatin formation was quantitated by ELISA (see Fig. 2). The *bars* and *error bars* represent the mean and S.E., respectively, of triplicate experiments.

Cofactor Requirements of Plasmin Reductase—The angiostatin-generating activity secreted by HT1080 cells was the highest of the cells examined and was used to further investigate the cofactor requirements of plasmin reductase. We previously reported that plasmin reductase secreted by HT1080 cells requires two components for activity, a protein component that can be heat-inactivated and a low M_r cofactor that can be GSH (11). The low M_r thiol compounds in HT1080cm were derivatized with the fluorescent compound 7-benzo-2-oxa-1,3-diazole-4-sulfonic acid and resolved by reverse-phase high performance liquid chromatography (24). The only low M_r thiol detectable in HT1080cm was GSH (not shown). The concentrations of GSH and GSSG were determined as described by Vandeputte *et al.* (25). The concentration of GSH in the HT1080cm was 1.1 ± 0.12 μM. This corresponded to secretion of 0.27 ± 0.03 nmol of GSH/10^6 cells/h. No GSSG was detected in HT1080cm.

To examine the GSH requirements of plasmin reductase, we measured the plasmin reductase activity of either Hepes-buffered saline or dialyzed HT1080cm supplemented with 0, 1, 5, or 10 μM GSH (Fig. 4). Dialysis of HT1080cm using a 12–14-kDa cut-off membrane reduced the angiostatin-generating activity to 12% of control. Supplementation of the dialyzed HT1080cm with 1 μM GSH doubled the angiostatin-generating activity, while 10 μM GSH restored the angiostatin-generating activity to that of undialyzed HT1080cm. This is in accordance with our previous findings (11). GSH at a concentration of 1 μM, the concentration in HT1080cm, had no angiostatin-generating activity on its own, while 10 μM GSH had 6% of the angiostatin-generating activity of undialyzed HT1080cm (Fig. 4).

The Kringle 5-Serine Proteinase Fragment of Plasminogen Is a Substrate for Plasmin Reductase—The plasminogen fragment K1–3, K1–4, K4, or K5-serine proteinase was incubated with HT1080cm for 2 h and then labeled with MPB. The fragment was resolved on 15% SDS-PAGE, transferred to PVDF membrane, and blotted with streptavidin-peroxidase to detect the MPB label. Of the plasminogen fragments, only K5-serine proteinase incorporated MPB (Fig. 5).

This observation suggested that the target disulfide bonds in plasmin for plasmin reductase resided in K5. Incubation of plasmin in pH 11 buffer causes reduction and isomerization of K5 disulfide bonds and results in formation of microplasmin (26, 27). Microplasmin has a Lys^{530} N terminus that is within K5. We compared the plasmin fragments generated by plasmin

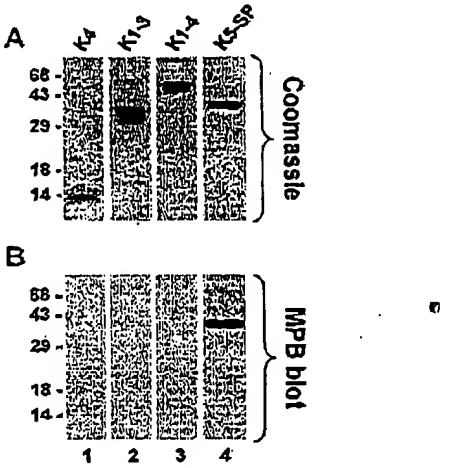

FIG. 5. **The kringle 5-serine proteinase fragment of plasminogen is a substrate for plasmin reductase.** *A*, fragments of plasminogen consisting of K4, K1–3, K1–4, and K5-serine proteinase were prepared by limited proteolytic digestion of plasminogen with porcine elastase and purified by a combination of lysine-Sepharose affinity and gel filtration chromatography (14). The plasminogen fragments (2 μg) were resolved on 15% SDS-PAGE under nonreducing conditions and stained with Coomassie Brilliant Blue. *B*, the plasminogen fragments (10 μg/ml) were incubated with HT1080cm for 2 h and then labeled with MPB. The fragments (0.2 μg) were resolved on 15% SDS-PAGE, transferred to PVDF membrane, and blotted with streptavidin-peroxidase to detect the MPB label. Of the plasminogen fragments, only K5-serine proteinase incorporated MPB. The positions of M_r markers are shown at left.

reductase with those generated by alkaline pH.

Comparison of Microplasmin Fragments Generated in either HT1080cm or in pH 11 Buffer—Plasmin was incubated in either 0.1 M glycine, pH 11 buffer or HT1080cm for 12 h at 37 °C. Samples were resolved and detected on gelatin zymography. Three major catalytically active plasmin fragments with apparent M_r of 40, 30, and 29 were generated in either pH 11 buffer or HT1080cm (Fig. 6*A*). The M_r 29 fragment corresponded to the M_r 29 microplasmin fragment described by Wu *et al.* (26, 27).

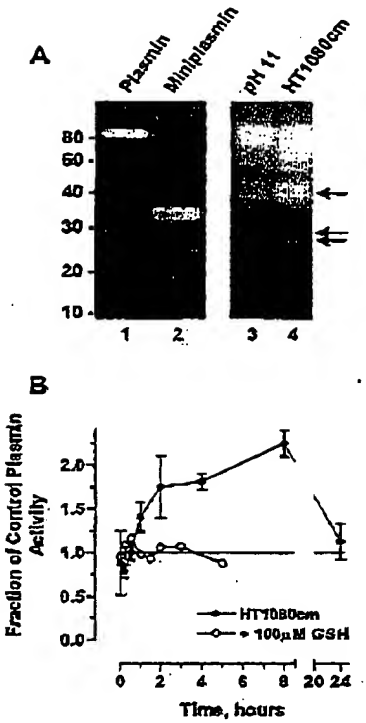

FIG. 6. **Comparison of microplasmin fragments generated in either HT1080cm or pH 11 buffer.** *A*, plasmin (10 μg/ml) was incubated in either 0.1 M glycine, pH 11 buffer (*lane 3*) or HT1080cm (*lane 4*) for 12 h at 37 °C. Samples corresponding to 0.2 μg of plasmin or fragments were resolved and detected on gelatin zymography. Matrix metalloproteinases in the HT1080cm (MMP-9 and MMP-2) were inactivated with EDTA. Intact plasmin (*lane 1*) and miniplasmin (*lane 2*) are shown for comparison. Fragments of apparent M_r of 40, 30, and 28 were observed. Note the close similarity in catalytically active plasmin fragments generated in either pH 11 buffer (*lane 3*) or HT1080cm (*lane 4*). Intact plasmin (*lane 1*) and miniplasmin (Val^{442}–Asn^{791}) (*lane 2*) are shown for comparison. The positions of M_r markers are shown at the *left*. *B*, plasmin (10 μg) was incubated with HT1080cm (1 ml) at 37 °C. At discrete time intervals, aliquots (20 μl) were taken and assayed for hydrolysis of H-D-Val-Leu-Lys-*p*-nitroanilide. Control activity was plasmin incubated with 20 mM Hepes, 0.14 M NaCl, pH 7.4 buffer at 37 °C. Plasmin was also incubated with 100 μM GSH in the Hepes buffer. Plasmin activity is reported as the fraction of control plasmin activity. The *data points* and *error bars* represent the mean and S.E., respectively, of three separate experiments.

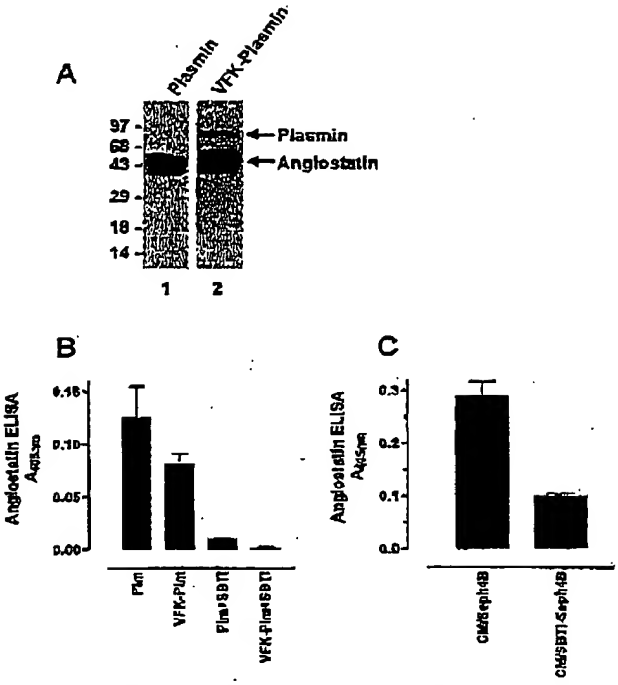

FIG. 7. **A serine proteinase other than plasmin was mostly responsible for proteolysis of reduced plasmin in HT1080cm.** *A*, generation of angiostatin from plasmin *versus* plasmin inactivated with VFK-CH_2Cl. Plasmin or VFK-plasmin (10 μg/ml) was incubated with HT1080cm for 2 h and then labeled with MPB. The fragments were resolved on 15% SDS-PAGE, transferred to PVDF membrane, and blotted with streptavidin-peroxidase to detect MPB-labeled angiostatin. Note that MPB labeled both intact VFK-plasmin and angiostatin fragments derived from the inactivated plasmin. The positions of M_r markers are shown at the *left*. *B*, plasmin or VFK-plasmin (10 μg/ml) was incubated in HT1080cm in the absence or presence of SBTI (25 μg/ml) for 2 h, and angiostatin formation was quantitated by ELISA (see Fig. 2). The *bars* represent the mean and range of duplicate experiments. *C*, HT1080cm (10 ml) was passed over either a Sepharose 4B or SBTI-Sepharose 4B column (2 ml). Plasmin (10 μg/ml) was incubated with the column eluate for 2 h, and angiostatin formation was quantitated by ELISA (see Fig. 2).

Microplasmin hydrolyzes the tripeptidyl *p*-nitroanilide substrate, H-D-Val-Leu-Lys-*p*-nitroanilide with 1.4-fold higher efficiency than plasmin. This is a consequence of a 1.4-fold increase in the catalytic constant (26). Plasmin was incubated in HT1080cm, and the initial rate of hydrolysis of H-D-Val-Leu-Lys-*p*-nitroanilide was measured at discrete time intervals (Fig. 6*B*). Plasmin activity is reported as the fraction of control plasmin activity. The efficiency of hydrolysis of the chromogenic substrate increased with time of incubation and peaked at ~2-fold enhanced efficiency at ~8 h. The initial rate of hydrolysis returned to control levels after 24 h of incubation.

Serine Proteinase(s) Other than Plasmin Were Mostly Responsible for Proteolysis of Reduced Plasmin in HT1080cm—Angiostatin is generated from reduced plasmin by a serine proteinase in CHO or HT1080cm (11). Serine proteinase activity is also required for generation of angiostatin from plasminogen or plasmin by cultured human prostate carcinoma cells (10). Plasmin autoproteolysis can account for angiostatin formation in the presence of protein reductants (11) or small thiols (12). To examine whether autoproteolysis is the operative mechanism in HT1080cm, we compared formation of angiostatin from either active plasmin or plasmin inactivated with Val-Phe-Lys-chloromethyl ketone (VFK-plasmin).

Plasmin or VFK-plasmin was incubated with HT1080cm for 2 h, and the angiostatin fragments were labeled with MPB. The MPB-labeled fragments were either resolved on SDS-PAGE and blotted with streptavidin-peroxidase (Fig. 7*A*) or quantitated by ELISA (Fig. 7*B*). Similar levels of angiostatin were generated from either active plasmin or VFK-plasmin, which indicated that autoproteolysis was not necessary for angiostatin formation. Interestingly, MPB labeled both intact VFK-plasmin and angiostatin fragments derived from the inactivated plasmin (Fig. 7*A*). This observation implied that reduction of plasmin preceded proteolysis. Formation of angiostatin from either active plasmin or VFK-plasmin was inhibited by the serine proteinase inhibitor, SBTI (Fig. 7*B*).

To examine the contribution of autoproteolysis of reduced plasmin to angiostatin formation, the HT1080cm was passed over either a Sepharose 4B or SBTI-Sepharose 4B column, and the angiostatin-generating activity of the eluate was measured (Fig. 7*C*). Sepharose 4B was used to control for nonspecific protein adsorption to the agarose matrix. Depletion of serine

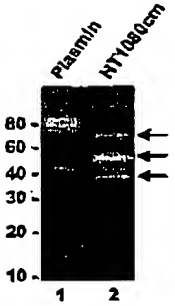

FIG. 8. **Profile of SBTI-binding serine proteinases in HT1080cm.** HT1080cm was passed over a SBTI-agarose column, and the bound proteinases were eluted with benzamidine. Serine proteinases from the equivalent of 3 ml of HT1080cm were resolved and detected using gelatin zymography. Proteinases with apparent M_r of 70, 50, and 39 were evident. For comparison, plasmin was incubated with HT1080cm for 12 h, and then 1 μg was resolved on gelatin zymography. Matrix metalloproteinases in the HT1080cm (MMP-9 and MMP-2) were inactivated with EDTA. Plasmin and catalytically active plasmin fragments were evident at apparent M_r of 85 and 40, respectively. The positions of M_r markers are shown at the *left*.

proteinases in HT1080cm reduced the angiostatin-generating activity to 34% of control.

SBTI-inhibitable serine proteinases in HT1080cm were purified on a SBTI-agarose column, and the bound enzymes were eluted with benzamidine. Serine proteinases from the equivalent of 3 ml of HT1080cm were resolved and detected using gelatin zymography (Fig. 8). Proteinases with apparent M_r of 70, 50, and 39 were evident. These enzymes did not correspond to plasmin or catalytically active plasmin fragments.

DISCUSSION

The formation of angiostatin from plasmin in the conditioned medium of transformed cells is a two-step process (11). First, one or more disulfide bonds in plasmin are reduced by a protein disulfide bond reductase, which we have called plasmin reductase, and a reductase cofactor, which can be a small thiol such as GSH. Second, reduced plasmin is cleaved by a serine proteinase producing angiostatin. Three angiostatin fragments are produced with apparent M_r of 45, 41, and 38 on nonreducing SDS-PAGE, which have the same Lys^{78} N terminus but different C termini.

Plasmin reductase activity was secreted by the human fibrosarcoma cell line HT1080; the human mammary carcinoma cell lines, MCF-7, MDA231, and BT20; and CHO cells. In contrast, the monocyte/macrophage cell line THP-1 did not secrete significant levels of plasmin reductase before or after stimulation with phorbol ester (not shown). Neither primary foreskin fibroblasts, blood monocyte/macrophages, nor macrovascular or microvascular endothelial cells secreted detectable plasmin reductase. In contrast, cultured bovine and rat vascular smooth muscle cells secreted small but reproducible levels of plasmin reductase. In general terms, cellular transformation appeared to be associated with secretion of plasmin reductase and angiostatin formation. This result suggested that angiostatin formation is driven by tumor cells *in vivo*. Production of angiostatin by vascular smooth muscle cells is an interesting observation and suggested that angiostatin may function in the atherosclerotic vessel wall. The metalloproteinase inhibitor EDTA did not have any effect on angiostatin production by the cell lines used in this study.

Gately *et al.* (12) have shown that a sufficient concentration of small thiols alone can generate angiostatin from plasmin. The concentration of GSH in the HT1080cm was 1.1 ± 0.12 μM, and it was the only small thiol detected in the medium. This

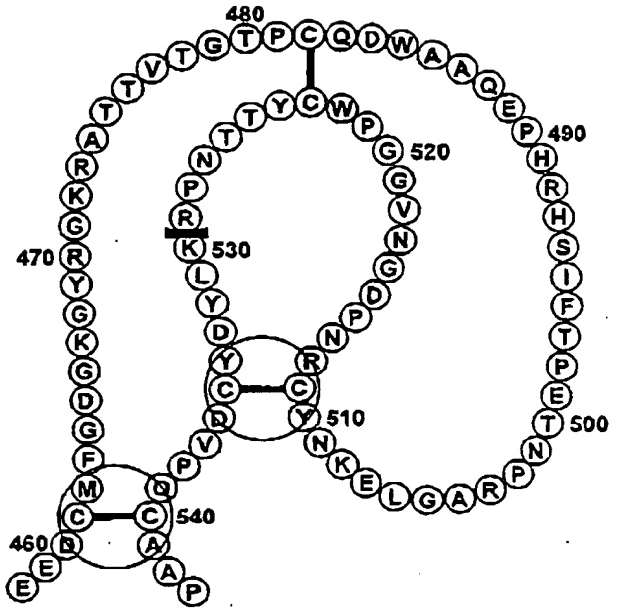

FIG. 9. **Model for generation of angiostatin by disulfide bond reduction and proteolysis in kringle 5 of plasmin.** We suggest that both the Cys^{461}–Cys^{540} and Cys^{611}–Cys^{535} disulfide bonds in plasmin kringle 5 are reduced by plasmin reductase (*shaded circles*). The reduced kringle 5 is then subject to proteolysis at either the Arg^{526}-Lys^{530} peptide bond (*solid black line*) or two other unidentified peptide bonds. Microplasmin is formed at pH 11 by autoproteolysis at the Arg^{560}-Lys^{630} peptide bond (26, 27).

corresponded to secretion of 0.27 ± 0.03 nmol of GSH/10^6 cells/h, which is comparable with the level of secretion of GSH by other cultured cells (28, 29). To examine the contribution of the GSH to the plasmin reductase activity of HT1080cm, the medium was dialyzed, and the angiostatin-generating activity was determined. Dialysis of HT1080cm reduced the angiostatin-generating activity to 12% of control. Supplementation of the dialyzed HT1080cm with 1 μM GSH doubled the angiostatin-generating activity, while 10 μM GSH restored the angiostatin-generating activity to that of undialyzed HT1080cm. In contrast, 1 μM GSH, the concentration in HT1080cm, had no angiostatin-generating activity, while 10 μM GSH had 6% of the angiostatin generating activity of HT1080cm. These results support our previous findings (11) and the proposal that plasmin reductase requires a small thiol cofactor such as GSH to provide the hydrogens and electrons to reduce the plasmin disulfide bonds. We suggest that high concentrations (100 μM) of small thiols have enough reducing power to reduce plasmin (12); however, these concentrations are not achievable in cell culture. Considered together, these observations implied that plasmin reduction in HT1080cm was catalyzed by plasmin reductase using GSH as a cofactor and not by GSH directly.

One or more disulfide bonds in the K5-serine proteinase fragment of plasminogen was reduced in HT1080cm. In contrast, neither K4, K1–3, nor K1–4 were substrates for plasmin reductase. This finding suggested that the target disulfide bond(s) for plasmin reductase were in K5. Plasmin undergoes autoproteolysis in alkaline pH, producing a catalytically active microplasmin fragment with a Lys^{630} N terminus (26, 27). Microplasmin consists of the last 10 amino acids of K5, the remaining 21 amino acids of the A chain, and the serine proteinase domain. Wu *et al.* (26, 27) noticed that both the Cys^{461}–Cys^{540} and Cys^{611}–Cys^{535} disulfide bonds in K5 must have

been reduced to release microplasmin from K1–4. They proposed that the increased $^{-}$OH ion concentration at pH 11 was responsible for reducing the two disulfide bonds. We observed that the proteinase fragments produced from plasmin in pH 11 buffer were of identical M_r to the proteinase fragments generated from plasmin in HT1080cm. Fragments with apparent M_r of 40, 30, and 29 were generated. The M_r 29 fragment is the same size as microplasmin. We suggest that the mechanism of plasmin proteolysis at pH 11 is the same as the mechanism of proteolysis in HT1080cm. In other words, plasmin reductase achieves at neutral pH what is achieved by $^{-}$OH ion at pH 11.

Three major angiostatin fragments (Fig. 1A) and three major serine proteinase fragments (Fig. 6A) were produced in HT1080cm. Based on the close similarity in plasmin fragments produced at alkaline pH and in HT1080cm, we suggest that at least the Cys^{461}–Cys^{540} and Cys^{511}–Cys^{535} disulfide bonds in K5 were reduced by plasmin reductase. For instance, reduction of only the K5 Cys^{482}–Cys^{523} and Cys^{511}–Cys^{535} disulfide bonds would not have resulted in release of angiostatin from plasmin. Similarly, angiostatin would have remained covalently linked to the remaining kringles if the Cys^{461}–Cys^{540} and Cys^{482}–Cys^{523} disulfide bonds were reduced, but not the disulfide bond at Cys^{511}–Cys^{535}. Reduction of the Cys^{461}–Cys^{540} and Cys^{511}–Cys^{535} disulfide bonds is consistent with all of the experimental data, although we cannot exclude reduction of the Cys^{482}–Cys^{523} disulfide bond in K5 or other disulfide bonds in K1–4.

The largest angiostatin fragment (M_r 45) and the smallest catalytically active fragment (M_r 29; microplasmin) probably resulted from cleavage at the Arg^{529}-Lys^{530} peptide bond. We hypothesize that cleavage can also occur at either the Arg^{479}-Ala^{474} or Arg^{503}-Ala^{504} peptide bond. Cleavage at these sites is favored by serine proteinases with plasmin-like specificity and would produce fragments of the size observed experimentally. Also, all three angiostatin fragments contained one or more cysteine residues (Fig. 1A). For the smallest angiostatin fragment (M_r 38) to contain a free thiol, proteolysis must have occurred C-terminal of Cys^{461}. A model of K5 and the proposed target disulfide bonds is shown in Fig. 9.

Gately *et al.* (10) observed that serine proteinase activity was required for generation of angiostatin from plasminogen or plasmin by cultured human prostate carcinoma cells, and we reported that serine proteinase activity was necessary for angiostatin generation by cultured CHO and HT1080 cells (11). Incubation of purified plasmin with the reductant thioredoxin (11), protein-disulfide isomerase (11), or high concentrations of small thiols (12) results in formation of angiostatin. This indicates that plasmin autoproteolysis can account for angiostatin formation. However, angiostatin was generated almost equally well from either active or inactive plasmin in HT1080cm, which implied that a proteinase(s) secreted by HT1080 cells was mostly responsible for plasmin proteolysis. The enzyme(s) was a serine proteinase, since it was inactivated by SBTI. Depletion of serine proteinases in HT1080cm by SBTI-agarose reduced the angiostatin-generating activity to 34% of control. This result implied that the rate of angiostatin formation is slowed when autoproteolysis of reduced plasmin is the operative mechanism. There were three major serine proteinases in HT1080cm with apparent M_r of 70, 50, and 39 that cleaved gelatin. One or more of these enzymes may contribute to generation of angiostatin from reduced plasmin. Therefore, although plasmin autoproteolysis can account for angiostatin formation, HT1080 cells did not rely entirely on this mechanism and secreted other serine proteinase(s) that cleaved reduced plasmin.

We have proposed that reduction of plasmin precedes proteolysis (11). This hypothesis was supported by the experiment that examined generation of angiostatin from inactivated plasmin. MPB labeled both intact VFK-plasmin and angiostatin fragments derived from the inactivated plasmin. This finding demonstrated reduction of disulfide bond(s) in intact plasmin, which supports our contention that plasmin reduction precedes proteolysis.

In summary, angiostatin formation involves disulfide bond reduction and proteolysis in K5 of plasmin. The motif in angiostatin responsible for its antiangiogenic properties is not known. Interestingly, the K5 domain of plasminogen has been shown to be a potent inhibitor of endothelial cell proliferation (30). Also, plasminogen interacts with cultured human umbilical vein endothelial cells through K5 (31). It is possible that the active motif in angiostatin resides in K5 and that the reduction and proteolytic events in this kringle expose the motif.

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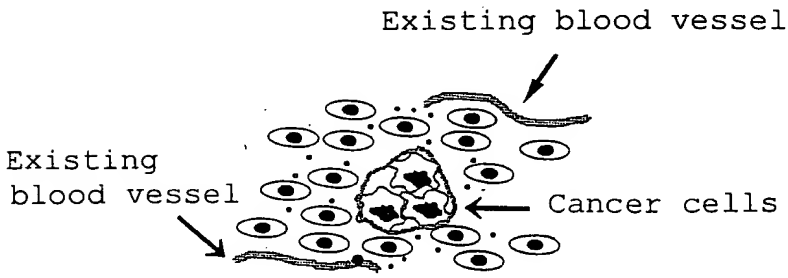
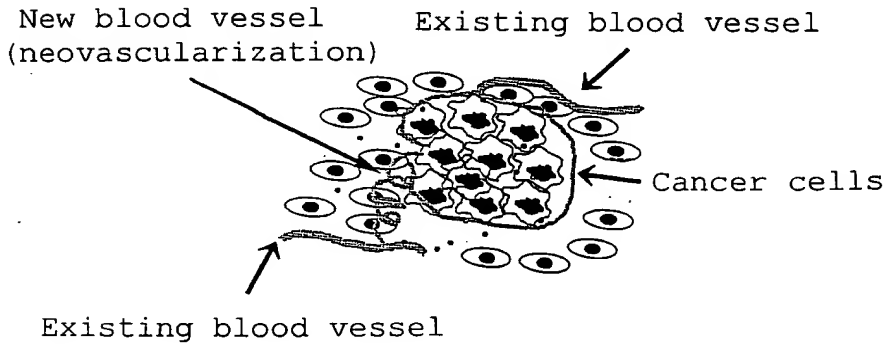
Mechanism of development of neovascularization and network structure thereof



Nutrients provision and waste material excretion

↓

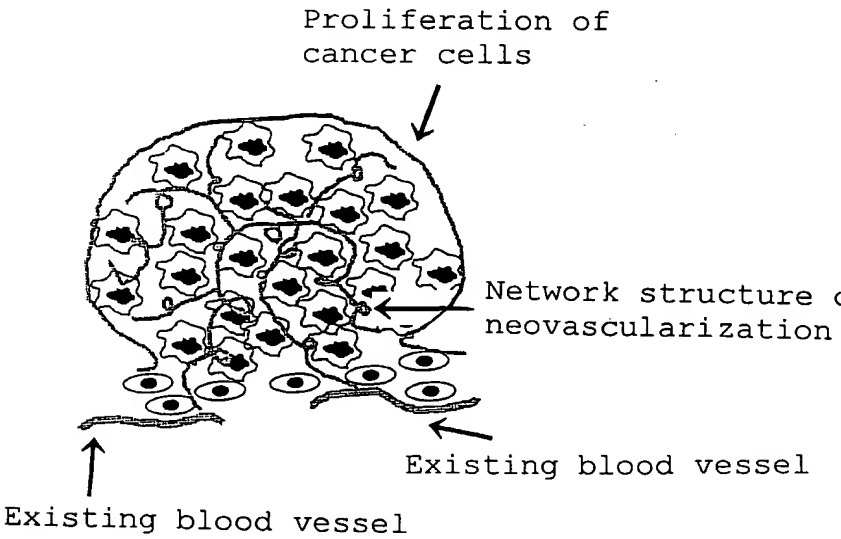
Metabolism through diffusion



For more proliferation, a new blood vessel for bypassing nutrients and waste material is necessary.

↓

Cancer cells release an active factor against existing blood vessels to introduce a new blood vessel (neovascularization).



Cancer cells acquiring a blood vessel rapidly proliferate and form a network structure of blood vessel as corresponding to their size.